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Proliferation and differentiation of normal breast epithelial cells are regulated by activation of the cellular tyrosine kinase machinery upon coordinated cellular stimulation through growth factor receptor tyrosine kinases and extra-cellular matrix receptor-induced activation of focal adhesion kinase FAK. This proposal is designed to investigate a novel hypothesis that Cbl provides, which has become established as a negative regulator of growth factor receptors, attenuates FAK-dependent growth signals in mammary epithelial cells. For this purpose, the Cbl interaction sites on FAK will be determined and the impact of mutations in these sites on the ability of FAK to mediate growth signals will be investigated. Given the recent findings that Cbl functions as ubiquitin ligase towards tyrosine kinases, we are examining the possibility that Cbl regulates FAK signaling by targeting it for degradation. The work reported here describes FAK mutants that appear to be unable to interact with Cbl tyrosine kinase-binding domain. Together with the generation of mutant forms of Cbl that are unable to mediate ubiquitination, these studies will directly establish if FAK is a target of Cbl. The present studies, thus, aim to define novel strategies to down-regulate proliferation signals in breast cancer cells.

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## Table of Contents

**Regulation of FAK Signaling in Mammary Epithelial Cells by Cbl Proto-Oncogene Product**

**Introduction:**

The experiments proposed in the application submitted for funding were designed to test a unique hypothesis that the proto-oncogene product Cbl down-regulates proliferation signals in mammary epithelial cells by concurrently targeting the focal adhesion kinase, FAK, in addition to its better defined ability to target growth factor receptors of the ErbB family. Genetic studies, initially in C. elegans and Drosophila systems and recently using mouse knock-outs, as well as extensive biochemical studies have established Cbl as a negative regulator of tyrosine kinases. At the time of this application, the tyrosine kinase-binding (TKB) domain of Cbl had been shown to be crucial for Cbl function, and initial pull-down experiments indicated that the TKB domain of Cbl interacts with FAK. These findings provided a rationale for the proposed analyses to identify the TKB domain binding sites on FAK and to introduce mutations in FAK to abrogate its ability to interact with the Cbl TKB domain in order to investigate the potential regulatory role of Cbl for FAK. Subsequent studies in our laboratory have also demonstrated that Cbl interacts with and regulates the function of Src-family kinases, which in turn are known to be important for activation of FAK. Notably, the regulation of Src-family kinases by Cbl involves at least two types of interactions, one dependent on the Cbl TKB domain and a second one involving binding between the proline-rich region of Cbl and the SH3 domain of Src-family kinases. Thus, it became clear that Cbl might regulate FAK both directly as well as indirectly via its negative regulatory effects on Src-family kinases. In keeping with these insights, we have sought additional evidence for the role of Cbl in FAK regulation using transfection approaches, and more importantly the recently derived cell lines from Cbl+/+ wildtype and Cbl-/- knockout mice. These findings have solidified our preliminary studies and provided a foundation for further studies to test our original hypothesis that Cbl controls FAK signaling in mammary epithelial cells. Understanding the mechanisms of this novel biochemical pathway to control mammary epithelial cell proliferation and migration is likely to provide crucial insights into breast cancer pathogenesis and may help in the design of newer forms of treatment.

**Modifications to Statement of Work:**

During the first year, we sought to further strengthen the basic rationale for our hypothesis. These studies, described below, clearly indicate a role for Cbl in regulating focal adhesion signaling and have established the association of Cbl and FAK in model cells. These analyses now set the stage for direct experiments using mammary epithelial cells. Furthermore, we have obtained a set of FAK mutants that are likely to be defective in Cbl TKB domain binding. While such studies were initially planned for the third year of the proposal, the availability of these reagents allows these studies to go forward immediately. Therefore, we propose a modified statement of work for next year.

The modified statement of work for the next year is:

1. Assess the physical and functional interactions between Cbl and FAK

A. Confirm Cbl-FAK association (previously established in Cos cells) in mammary epithelial cells
B. Establish conditions of co-stimulation of mammary epithelial cells with extra-cellular matrix and EGF; compare FAK phosphorylation and cell migration response to these treatments between control mammary epithelial cells and wildtype or mutant Cbl-transfected cells.
C. Establish additional mutant Cbl transfectants of MECs, in particular the G306E Cbl transfectants; carry out analyses as in 1A and 1B.

2. Generate mammary epithelial cells and reconstituted fibroblasts expressing FAK mutants to test the role of Cbl in FAK down-regulation

A. Introduce wildtype FAK and its mutants into FAK-/- fibroblasts and mammary epithelial cells; demonstrate the expression of introduced proteins.
B. Assess the association of FAK mutants with Cbl.
C. Assess the phosphorylation of FAK and its mutants upon stimulation as in 1B.

**Body:**

Studies carried out in the current reporting period helped further the training of the trainee, and resulted in a number of novel findings that strengthen our proposed hypothesis and provide a basis for further studies. Furthermore, the reagents characterized over the last year as well as the technical procedures that have been established will facilitate the achievement of our original goals.

Initial studies using a GST fusion protein had indicated that the Cbl TKB domain could directly bind to FAK. To further investigate the role of the TKB domain in Cbl-FAK association, we have carried out analyses in two different cell systems. In the first set of experiments, expression constructs encoding hemagglutinin (HA) epitope tagged Cbl or its mutants and FAK were cotransfected into Cos7 cells. Immunoblot analysis of whole cell lysates demonstrated the expression of various exogenously introduced proteins: FAK, full-length Cbl or its G306E mutant, and Cbl-N (the TKB domain) or its G306E mutant (Fig. 1). Anti-FAK immunoprecipitations were carried out from the same lysates followed by immunoblotting with anti-HA antibody to detect the association between FAK and various Cbl proteins. As expected from GST fusion protein analyses, Cbl-N prominently co-immunoprecipitated with FAK (Fig. 1, lane 6) and this association was severely impaired by G306E mutation of the Cbl-N construct (lane 7). Importantly, a small proportion of full-length Cbl also co-immunoprecipitated with FAK (lane 4) but the G306E mutation of Cbl did not reduce the association; if anything, a higher level of association was noted (Fig. 1, lane 5 versus 4). Co-immunoprecipitation with FAK was also observed using the 70Z3 mutant of Cbl, which has a 17-amino acid deletion of a17-amino acid region (residues 366-382) adjacent to N-terminal boundary of the RING finger domain. This mutation converts the negative regulatory protein Cbl into a potent oncogene and is accompanied by the loss of the ubiquitin ligase activity of Cbl. Thus, these results in a transient transfection system confirmed the ability of the Cbl TKB domain to associate with FAK in a manner that requires an intact Cbl SH2 domain, indicating that Cbl TKB domain recognizes a

phosphotyrosine-containing motif on FAK. Notably, however, these results also indicate an important role of other (non-TKB) mechanisms in FAK-Cbl association. To further confirm the results of transient transfection experiments, NIH-3T3 cells stably transfected with HA-tagged wildtype Cbl were analyzed. Anti-FAK immunoblotting of anti-HA immunoprecipitates, but not of the control anti-CD8 immunoprecipitates, showed co-immoprecipitated endogenous FAK. Conversely, anti-HA immunoblotting of anti-FAK immunoprecipitates, but not the control normal rabbit serum immunoprecipitates, showed coimmunoprecipitation of HA-tagged Cbl. Notably, the association was seen both in control and fibronectin-stimulated cells; however, an increase in the phosphorylation of Cbl-associated FAK was observed. In view of the complex mechanism of association between Cbl and FAK suggested by above analyses, we wished to obtain additional evidence for the likelihood of our hypothesis prior to embarking on mutagenesis of FAK and stable transfections in mammary epithelial cells and FAK-/- cells. Through recent work in the laboratory, a pair of matched primary embryo fibroblast cells from Cbl+/+ and Cbl-/- mice has been obtained (Andoniou et al. Molecular and Cellular Biology, 20:851-867, 2000). Therefore, we used these cells to investigate whether Cbl was likely to participate in focal adhesion signaling.

Morphologic examination of Cbl+/+ and Cbl-/- cells showed that Cbl-deficient cells lacked the long processes typical of wildtype cells and instead exhibited short substratum-associated structures and a dramatically more spread out cell body (Fig. 3). These changes indicated a change in cytoskeletal architecture and/or focal adhesions. To investigate this possibility, cells were plated on fibronectin-coated coverslips overnight, followed by two-color immunofluorescence staining using FITC-conjugated pahalloidin to stain f-actin and anti-paxillin antibody followed by phycoerythrin-conjugated secondary antibody to visualize focal adhesions. As expected, Cbl+/+ cells exhibited a fine meshwork of actin primarily in the sub-membranous area with fine stress fibers; paxillin staining of these cells demonstrated small more peripherally located focal adhesions (Fig. 4, top panel). In contrast, identically plated Cbl-/- cells exhibited markedly enhanced actin fibers (stress fibers) and vastly increased size and density of focal adhesions, which were distributed throughout the cell body (Fig. 4, lower panel). The presence of an increased number and size of focal adhesions, together with increased actin stress fibers, suggests a likely defect in focal adhesion dynamics in these cells. Notably, this phenotype is reminiscent of that observed in FAK-deficient cells (Ilic D, et al. Nature 377: 539, 1995). Therefore, we hypothesized that the absence of Cbl in these cells may prevent the turnover of focal adhesions, possibly by failing to induce the degradation of FAK or another component of focal adhesions. If this was the case, then a defect in directed cell migration might be expected in Cbl-/- as compared to Cbl+/+ cells. To test this possibility, we developed a quantitative cell migration assay by introducing a critical modification of a previously described protocol, involving immunofluorescence visualization of migrated cells after staining with propidium iodide (Fig. 5).

Using the modified cell migration assay, we compared the basal and PDGF-induced migration of Cbl+/+ and Cbl-/- cells. As is seen in Figure 6 (bottom panel), Cbl-/- cells show a marked reduction of cell migration under both conditions. The upper panel shows a histogram plot of these data. To further investigate the observed defect in cell migration in Cbl-/- cells, we tested their migration towards lysophospatidic acid (LPA) a major component of serum responsible for cell proliferation and migration, which is known to activate focal adhesion

signaling. As shown in Fig. 7 (upper panel), LPA induced a dose-dependent migration of Cbl+/+ cells, but Cbl-/- cells exhibited a sever defect of LPA-induced migration. Similarly, a defect in serum-induced migration was seen with Cbl-/- cells (Fig. 7, lower panel).

Given the essential role of Src-family kinases in FAK-dependent cell migration (Klinghoffer RA, et al. EMBO J. 18:2459-2471, 1999), and the ability of Cbl to downregulate Src-family kinases (Andoniou et al. Molecular and Cellular Biology, 20:851-867, 2000), we have also begun to ask if Cbl might influence FAK signaling indirectly, via ubiquitin-dependent degradation of Src-family kinases. In collaboration with members of the laboratory, we have now demonstrated that Cbl indeed induces the ubiquitination-dependent degradation of a prototype Src-family kinase Fyn and as a result markedly reduces the downstream activation events induced by activated Src-family kinases (Rao N, et al. Manuscript submitted; attached as Appendix Manuscript). If upon further analysis a direct in vivo interaction between Cbl and FAK is not demonstrated as the mechanism of Cbl regulation of FAK, then the Src-family kinase-mediated mechanism will become the primary focus of investigation.

Recently, we have initiated a collaborative investigation with Dr. Wen-Cheng Xiong (University of Alabama, Birmingham, Alabama) and Dr. J. Thomas Parsons (University of Virgina, Charlottesville, VA) to identify the Cbl TKB domain-binding sites of FAK. Using GST-Cbl-N fusion protein that we provided to these investigators, they determined that two known phosphorylation sites within FAK, Y397 and Y861 could mediate in vitro binding. Notably, however, Y861 appears to be the predominant site of binding in vivo. We have obtained the following vectors encoding FAK and its mutants, all with a Myc tag from Drs. Xiong and Parsons: FAK wildtype; FAK-Y397F; FAK-Y861F; and FAK-Y397/861F. In initial experiments, we assessed the expression of these FAK proteins upon transfection into 293T human embryonic kidney cells. In each case, transfection of increasing amounts (0.1 to 3 μg) of pCMV-based expression constructs led to increasing expression of a stable anti-Myc antibody-reactive protein of the correct size, as assessed by immunoblotting of whole cell lysates (Fig. 8). In additional analyses, we assessed the ability of a Src-family kinase Fyn to induce the phosphorylation of these FAK proteins. 293T cells were transfected with the indicated FAK construct, together with increasing amounts of activated form of Fyn (Fyn-Y528F), and equal aliquots of cell lysates were analyzed by anti-phosphotyrosine immunoblotting. As is evident from Fig. 9, wildtype FAK was efficiently phosphorylated with maximal phosphorylation even with the lowest amount of transfected Fyn. Both of the single tyrosine mutants (Y397F and Y861F) showed moderate reduction in phosphorylation, whereas the double mutant showed a marked reduction in Fyn-induced phosphorylation. Further characterization of the interaction of these mutants with Cbl is planned (see statement of work for next year).

Overall, the additional studies of Cbl-FAK association in model cells, together with the dramatic alterations of focal adhesions in Cbl-/- cells strongly support our original hypothesis that Cbl regulates FAK and influences cell proliferation in response to co-stimulation by extra-cellular matrix and growth factors. Studies proposed in the next year are designed to test this hypothesis directly.

**Key Research Accomplishments:**

- Established the in vivo association between Cbl and FAK and identified a TKB-dependent and a TKB-independent mechanism for such association.
- Obtained FAK mutants with potential defects in Cbl binding and partially characterized these.
- Established a modified cell migration assay to assess the role of Cbl in FAK-dependent cell migration.
- Demonstrated a defect in PDGF, LPA and serum-induced cell migration in Cbl-/- cells as compared to Cbl+/+ cells.
- Demonstrated altered focal adhesions and actin stress fibers in Cbl-/- cells as compared to Cbl+/+ cells.
- Demonstrated the role of Cbl ubiquitin ligase activity in the regulation of Src-family kinase Fyn, which is upstream of FAK in integrin-dependent focal adhesion signaling.

**Reportable Outcomes:**

**Publications:**

- Navin Rao, Amiya K. Ghosh, Pengcheng Zhou, Satoshi Ota, Christopher E. Andoniou, **Patrice Douillard** and Hamid Band. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. Submitted.

**Reagents:**

- Characterized a panel of FAK mutants.
- Cbl-/- cells with defective cell migration response.

**Funding applied for based on this work:**

The work carried out under this award will be part of the background and preliminary studies for an NIH RO1 renewal application (Regulating Tyrosine Kinase Signals in Breast Cancer) that is being submitted by the applicant's mentor, Dr. Band, later this year.

**Manuscripts included:**

- Navin Rao, Amiya K. Ghosh, Pengcheng Zhou, Satoshi Ota, Christopher E. Andoniou, **Patrice Douillard** and Hamid Band. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. Submitted.

**Conclusions:**

In conclusion, our results in model cell systems demonstrate an interaction between Cbl and FAK, and we demonstrate altered actin cytoskeleton and focal adhesions in Cbl-/- cells, strengthening our hypothesis that Cbl is involved in the regulation of FAK signaling. Given the critical role of FAK signaling in growth factor and extra-cellular matrix-dependent cellular proliferation and differentiation, the findings presented here are likely to be of general significance for other cell types. Importantly, the reagents developed here now allow us direct analyses to elucidate the role of Cbl in regulating the FAK signaling in mammary epithelial cells.

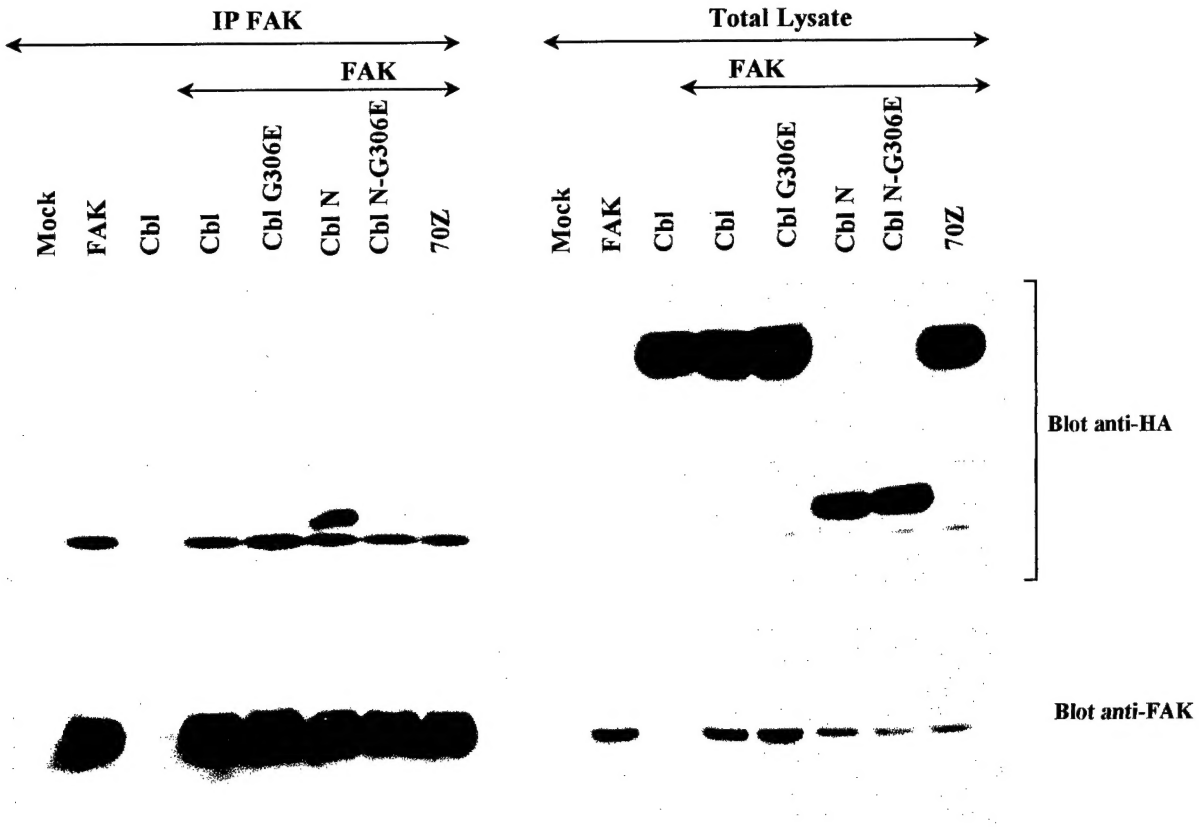



**Fig. 1. Cbl-FAK association in vivo in Cos-7 cells.** Cos-7 cells were transfected with pAlterMAX vector constructs encoding the indicated Cbl proteins with an N-terminal HA tag, together with a pCDNA3 vector encoding FAK, using the calcium phosphate co-precipitation method. Mock transfectants received vector only. Triton X-100 lysates were prepared 60 hours post-transfection and subjected to immunoprecipitation with an anti-FAK antibody (IP FAK) and resolved on a SDS-PAGE gel. Aliquots of whole cell lysates were concurrently resolved. Following transfer to PVDF membranes, serial anti-HA (to detect introduced Cbl) and anti-FAK immunoblotting was carried out. Upper and middle panels represent the appropriate segments of the anti-HA blot to show full-length Cbl versus Cbl-N proteins.

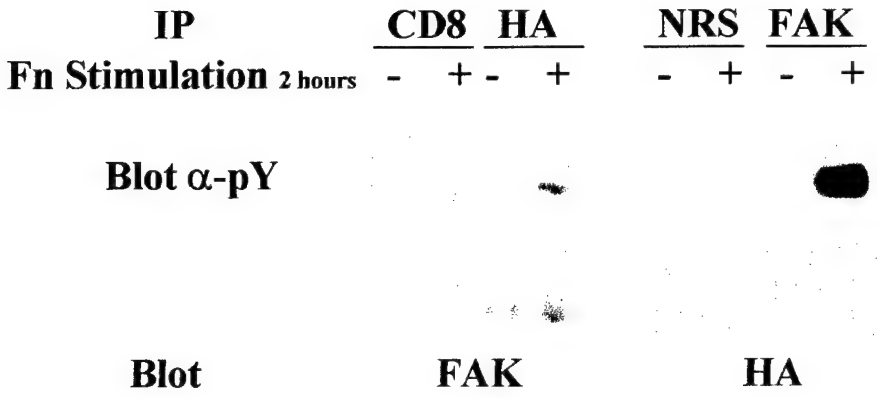



**Fig. 2. Cbl-FAK association in vivo in NIH-3T3 cells.** 3T3-HA-Cbl.8 (a clone of NIH-3T3 cells stably expressing HA-tagged Cbl) cells were plated on dishes coated with 10 μg/ml poly-L-lysine (Fn, -) or fibronectin (Fn, +). After 2 hours, Triton X-100 lysates were prepared and subjected to immunoprecipitation (IP) with anti-HA monoclonal antibody (12CA5) (with anti-CD8 as control) or anti-FAK polyclonal antibody (with normal rabbit serum [NRS] as control). IP's were immunoblotted first with anti-phosphotyrosine (pY) antibody 4G10 (upper panels) followed by ant-FAK or anti-HA antibodies as indicated (bottom panels). Note that phospho-tyrosine signals on total FAK as well as Cbl-associated FAK increase with fibronectin stimulation; however, the level of co-immunoprecipiation between FAK and Cbl was unchanged.

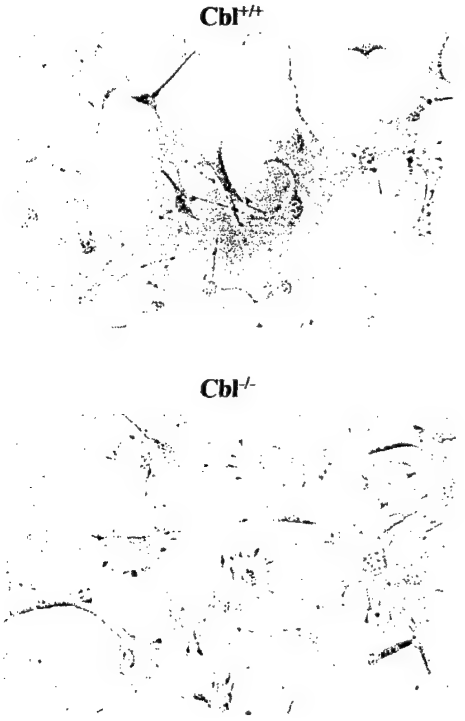



**Fig. 3. Morphological alterations in Cbl-/- primary embryonic fibroblasts**. Primary embryonic fibroblast lines derived from Cbl+/+ and Cbl-/- mice were cultured under regular growth conditions (DMEM with 10% fetal bovine serum) for 24 hours before taking the phase contrast pictures (250X magnification). Note that Cbl-/- cells lack the long processes typical of wildtype cells and instead exhibit short substratum-associated structures and a more spread out cell body.

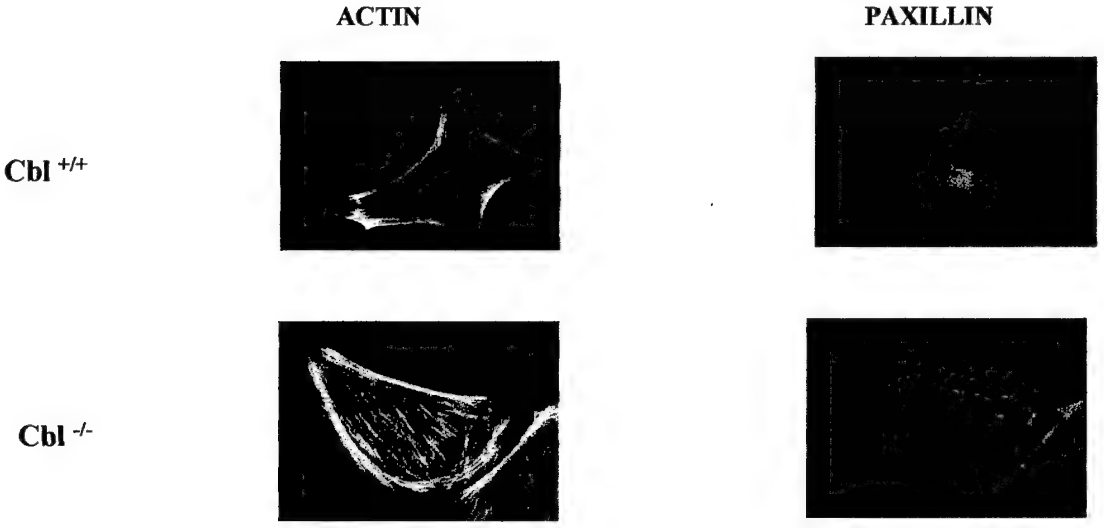
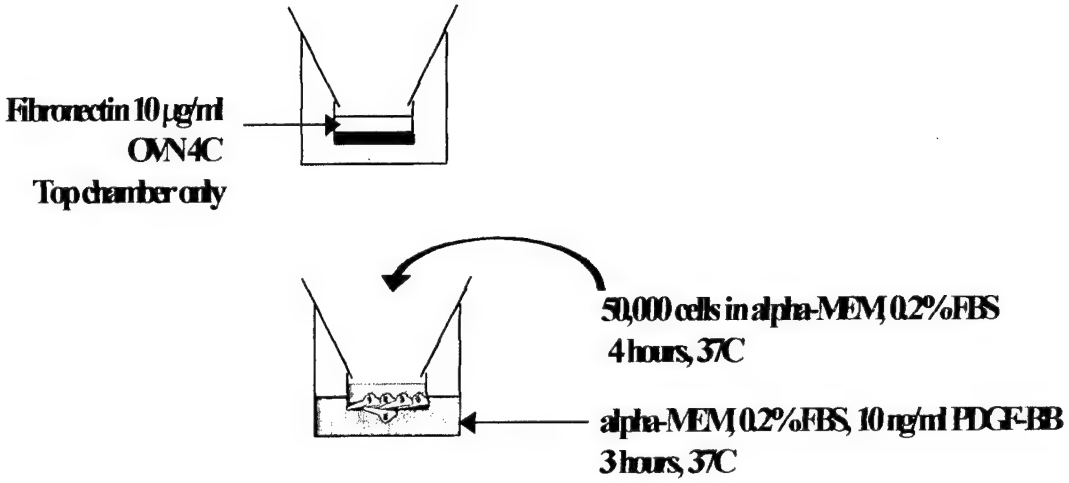
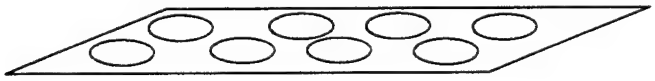



**Fig. 4. Increased actin stress fibers and focal adhesions in Cbl-/- primary embryo fibroblasts.** Glass coverslips were coated with 10 μg/ml fibronectin overnight at 4°C followed by blocking with 1% bovine serum albumin. Cbl+/+ and Cbl-/- primary embryo fibroblast cells were then plated on the fibronectin-coated coverslips for 48 hours, fixed in 3.8% paraformaldehyde in PBS. Cells were then double stained with FITC-labeled phalloidin (which stains f-actin) and anti-paxillin antibody (which stains focal adhesions). Paxillin staining was visualized with phycoerythrin-conjugated secondary antibody.

# Chemotaxis Assay Protocol




Wash 2X PBS
Fix in MetOH for 20 min at -20C
Air dry
Stain with Propidium Iodide and RNAse A for 30 min in PBS D-Glucose in the bottom chamber
Wash once with PBS D-Glucose
Remove cells on the top
Cut membrane and place it on a slide



Take pictures (100X) and count cells

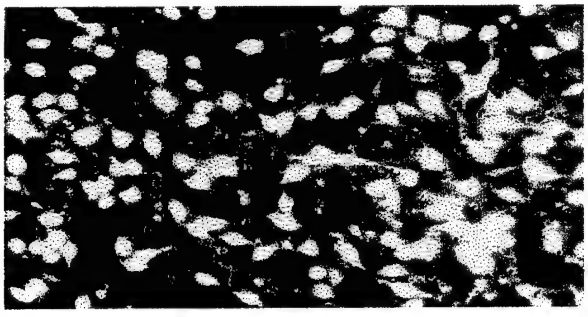
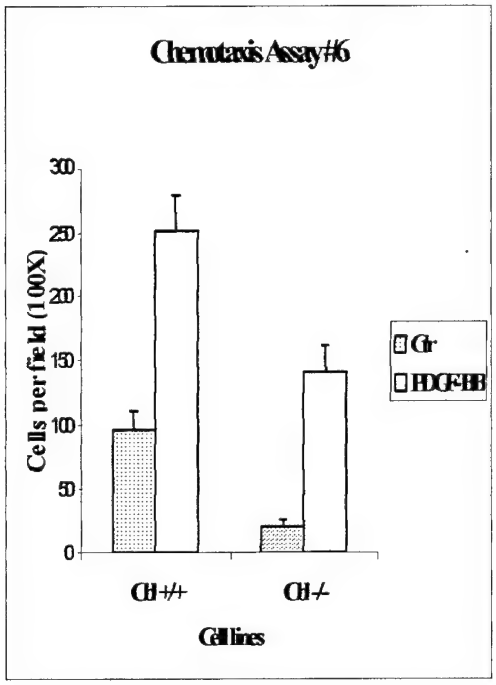


**Fig. 5. Modified Boyden chamber cell migration assay.** Details of the protocol are indicated. The lower panel shows a fluorescence picture of migrated Cbl+/+ primary embryo fibroblasts.



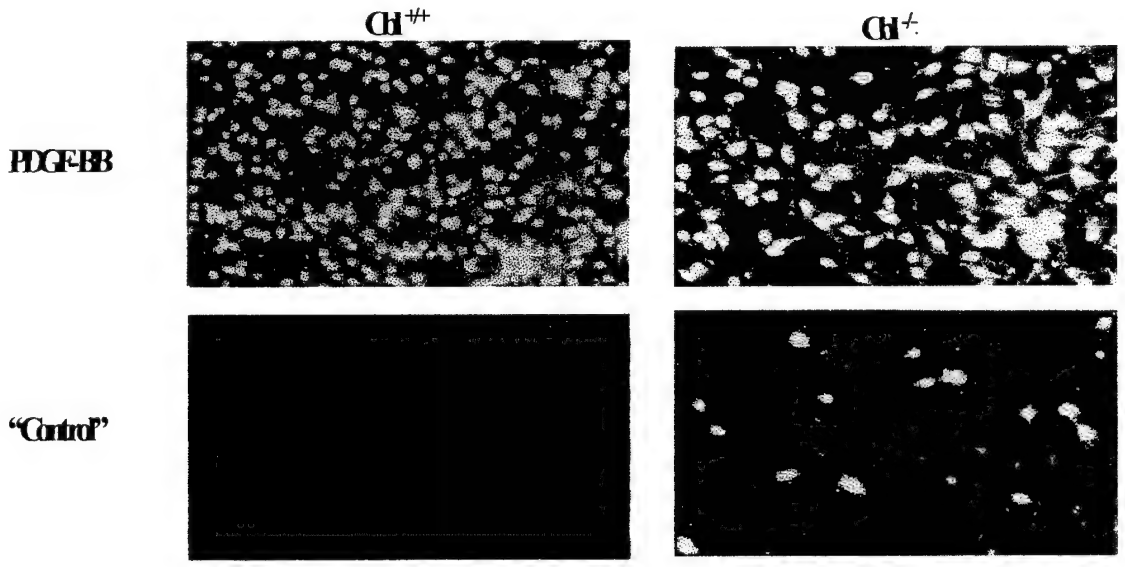




**Fig. 6. Reduced basal and PDGF-induced migration of Cbl-/- primary embryonic fibroblasts.** The assay was performed as in described in Fig. 5 and migrated cells were counted. PDGF-BB was used at 20 ng/ml final concentration. A representative assay is shown. Each par represents triplicates.

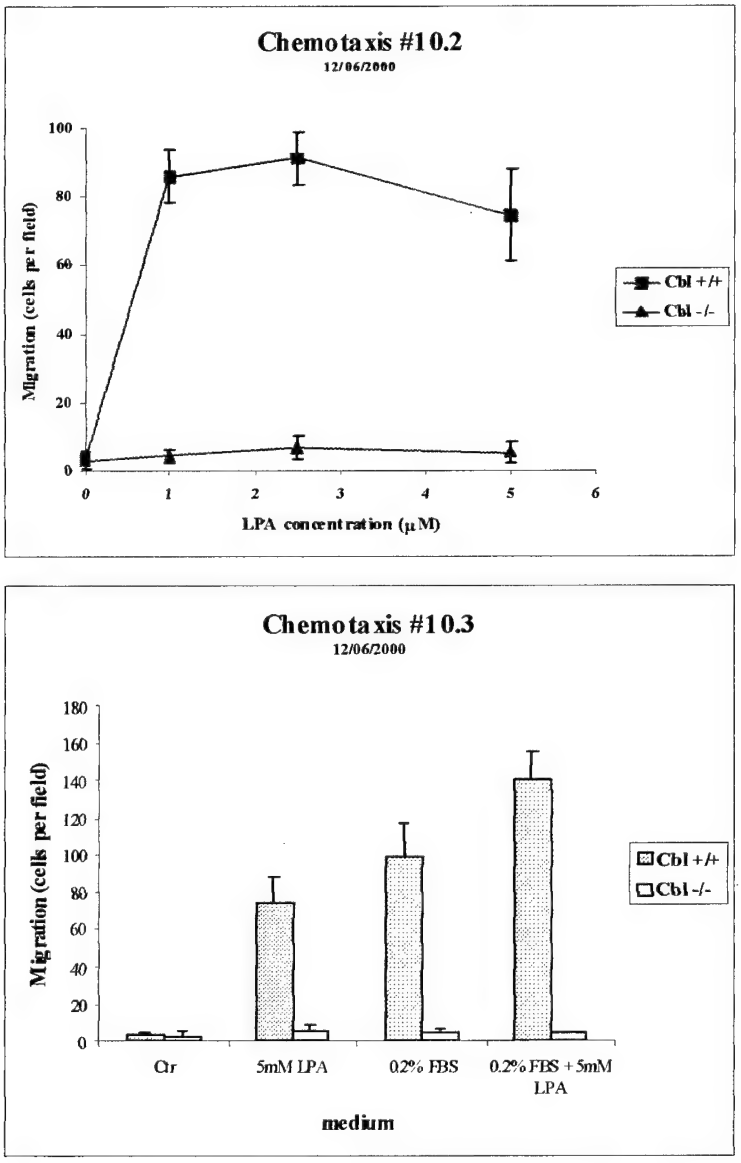



**Fig. 6. Reduced migration of Cbl-/- primary embryonic fibroblasts in response to lysophospatidic acid (LPA) and fetal bovine serum (FBS).** The migration assays were performed in triplicates as described in Fig. 5 using PDGF-BB was used at 20 ng/ml final concentration. A representative assay is shown. Each par represents triplicates.

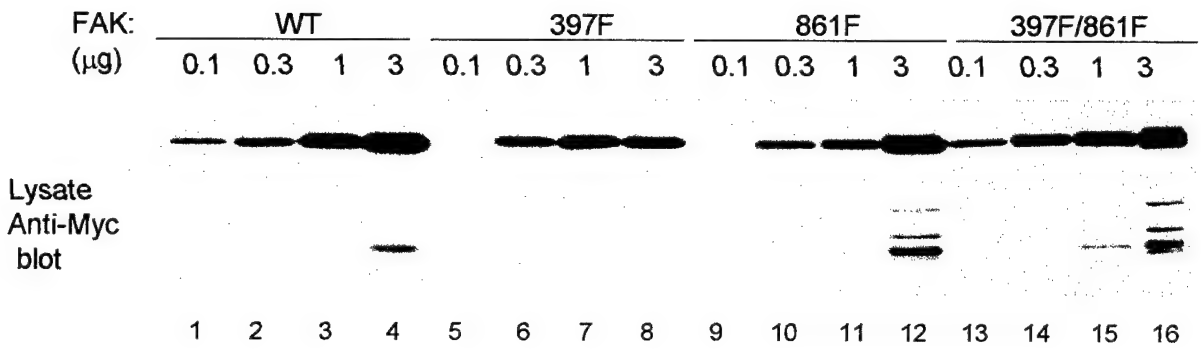



**Fig. 8. Expression of various FAK mutants.** 293T cells were transfected with the indicated amounts of pCMV constructs encoding Myc-tagged wildtype FAK or its Y->F mutants. 40 hours post-transfection, Triton X-100 lysates were prepared and equal aliquots of lysates were resolved by SDS-PAGE followed by anti-Myc (9E10 antibody) immunoblotting.

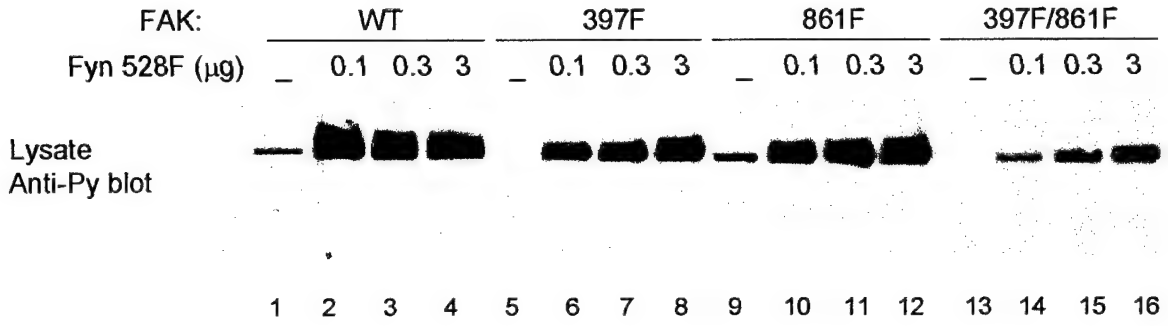



**Fig. 9. Phosphorylation of various FAK mutants.** 293T cells were transfected with 1 μg of pCMV constructs encoding the indicated FAK protein together with increasing amounts of a pAlterMAX construct encoding activated Fyn tyrosine kinase (Fyn-Y528F). 40 hours post-transfection, Triton X-100 lysates were prepared and equal aliquots of lysates were resolved by SDS-PAGE followed by anti-phosphotyrosine (4G10 antibody) immunoblotting. Equal levels of various FAK proteins were confirmed by anti-FAK immunoblotting (not shown).

**An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn**

Navin Rao¶, Amiya K. Ghosh§, Pengcheng Zhou, Satoshi Ota&, Christopher E. Andoniou§#, Patrice Douillard§, and Hamid Band**

Lymphocyte Biology Section, Division of Rheumatology, Immunology and Allergy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

**Running Title: Cbl-dependent ubiquitination of Fyn**

**SUMMARY**

Members of the Cbl family of proteins have emerged as evolutionarily conserved negative regulators of protein tyrosine kinases. Recent evidence indicates that the negative regulation of activated receptor tyrosine kinases is mediated by Cbl-dependent ubiquitination and subsequent lysosomal targeting. Here, we have investigated the role of Cbl ubiquitin ligase activity in the negative regulation of a non-receptor tyrosine kinase, the Src-family kinase Fyn. Using primary embryonic fibroblasts from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, we demonstrate that endogenous Cbl mediates the ubiquitination of Fyn and determines the rate of Fyn turnover. By analyzing CHO-TS20 cells, with a temperature-sensitive ubiquitin activating enzyme, we demonstrate that intact cellular ubiquitin machinery is required for Cbl-induced degradation of Fyn. Analyses of Cbl mutants, with mutations in or near the RING finger domain, in 293T cells, and *in vitro* ubiquitination assays revealed that the ubiquitin ligase activity of Cbl is essential for Cbl-induced degradation of Fyn by the proteasome pathway. Finally, use of a SRE-luciferase reporter demonstrated that Cbl-dependent negative regulation of Fyn function requires the region of Cbl that mediates the ubiquitin ligase activity. Given the conservation of structure between various Src family kinases and the ability of Cbl to interact with multiple SFKs, we propose that Cbl-dependent ubiquitination provides a general mechanism to negatively regulate activated SFKs.

## INTRODUCTION

Src-family kinases (SFKs) constitute a large family of evolutionary conserved protein tyrosine kinases (PTKs) that mediate crucial biological functions, including critical roles in tissue and organ development, cell differentiation, adhesion and migration, mitogenesis, and immune responses (1-3). Given the ease with which mutations can render them dominantly oncogenic (4), SFKs have served as an important model to understand the mechanisms that ensure the precise regulation of PTKs.

All SFKs share a conserved domain structure, consisting of a membrane-anchoring N-terminal myristoylation signal, adjacent SH3 and SH2 domains, a kinase domain, and a tyrosine residue near the C-terminal tail whose phosphorylation by the C-terminal Src kinase (CSK) is required for repression (1,2). The crystal structures of Src and Hck proteins, together with a large body of mutational analysis, have established a general paradigm for how the repressed state of a SFK is achieved and have suggested potential mechanisms of activation (5,6). Intra-molecular binding of the SH3 domain to a type II polyproline-like helix within the SH2-kinase linker region and the SH2 domain binding to the conserved C-terminal phosphotyrosine residue force the kinase domain into an inactive conformation (5,6). It is hypothesized that activation signals lead to displacement of the SH2 and SH3 domains from their intra-molecular ligands, promoting the open, active, conformation of the kinase domain and concurrently releasing the SH2 and SH3 domains for assembly of signaling complexes. Consistent with this model, inactivating point mutations in the SH3 or SH2 domains can significantly enhance the kinase activity (7). Furthermore, mutations within the SH2-kinase linker that abolish intra-molecular binding of the SH3 domain, or overexpression of high affinity SH3 domain-binding ligands, results in increased kinase activity of Hck, Src, or Lck (8-10). Similarly, deletion or substitution of the negative regulatory tyrosine within the carboxyl tail of SFKs results in enhanced kinase activity and oncogenesis (11), and deletion of the CSK gene leads to constitutively activated SFKs (12,13). Conversely, substitutions that enhance the affinity of the C-terminal phosphotyrosine motif for the SH2 domain decrease the kinase activity (14).

While the above paradigm elegantly accounts for basal repression and provides a plausible scheme for activation of SFKs, it is not clear at present if and how the activated SFKs are returned to their basal repressed conformation. Given recent evidence that SFKs require cellular chaperones, such as members of the HSP90 family, for proper folding (15), it is likely

that cells utilize additional mechanisms for deactivation of SFKs and, by implication, other PTKs. Without such ancillary mechanisms, activated SFKs could accumulate resulting in deleterious consequences for a cell. Recent studies have demonstrated that the proto-oncoprotein Cbl provides one such mechanism for deactivation of SFKs (16,17).

Cbl is a member of an evolutionary conserved family of cytoplasmic proteins that have emerged as negative regulators of several PTKs (18,19). Genetic analyses in *Caenorhabditis elegans* and *Drosophila* have established the Cbl homologues in these species as negative regulators of various EGFR-mediated developmental pathways (20-22). Furthermore, genetic ablation of murine Cbl produced hypercellularity and altered development of several organ systems (23,24), whereas Cbl-b deletion led to hyperproliferation and hyperactivation of immune cells resulting in autoimmunity (25,26). Studies of receptor tyrosine kinases (RTKs) have demonstrated that Cbl enhances the efficiency with which ligand-activated receptors are targeted for lysosomal degradation (27-29). Notably, this effect of Cbl directly correlates with its ability to enhance the ubiquitination of unknown sequences within these receptors (28,30-34). By analogy with yeast membrane receptors, whose lysosomal targeting is dependent on ubiquitination (35), it has been proposed that Cbl-dependent ubiquitination of RTKs may serve as a signal for lysosomal sorting. The mechanism of this sorting and the potential role of ubiquitination in targeting RTKs for proteasomal degradation are less clear. Notably, Cbl-dependent negative regulation of non-receptor PTKs Syk and ZAP-70 has demonstrated that Cbl targets their activated pools for degradation (36-38). However, it is not yet known if Cbl-induced degradation of Syk or ZAP-70 is mediated via the ubiquitin-proteasome pathway, although structure function analyses of Cbl are consistent with this possibility.

A highly conserved N-terminal region, corresponding to Cbl sequences found in oncogenic *v-Cbl*, specifically interacts with negative regulatory phosphorylation sites within Syk/ZAP-70 and EGFR tyrosine kinases, providing a basis for the selective recruitment of Cbl to activated pools of these PTKs (27,36,39). Mutations (in Cbl or its target PTKs) that abrogate Cbl tyrosine kinase binding (TKB) domain interaction with PTKs block Cbl-dependent negative regulation of epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGFR) and Syk/ZAP-70 PTKs (27,30-32,34,36,37). The crystal structure of Cbl's N-terminal TKB domain, together with its cognate binding site phosphopeptide in ZAP-70, revealed an integrated

phosphotyrosine-binding platform composed of a four-helical bundle, an EF hand and an incomplete SH2 domain (40).

Further mutational analyses have demonstrated that the RING finger domain of Cbl, which is the most highly conserved domain through evolution, is required for negative regulation of EGFR, PDGFR, Syk and ZAP-70, as well as for ubiquitination of the EGFR (27,28,31,32,34,37,41). Importantly, the N-terminal half of Cbl, comprised of only the TKB and RING finger domains, was capable of inducing the negative regulation of Syk or EGFR, as well as the ubiquitination of EGFR (30,34,38). Recent data have demonstrated that the RING finger domain binds to, and possibly helps to activate, members of the ubiquitin conjugating enzyme (UBC) family (E2s), thus establishing the N-terminal half of Cbl as a PTK-directed ubiquitin ligase (E3) (42). The C-terminal half of Cbl contains a proline-rich region for association with SH3 domain-containing proteins, multiple tyrosine phosphorylation sites that provide docking sites for SH2 domain-containing proteins, and a leucine zipper that may mediate dimerization (18,43). The functional significance of these additional protein-protein interactions has not been fully clarified.

Recently, we demonstrated that overexpression of Cbl in a 293T cell system led to degradation of the activated pool of Fyn, a prototype SFK, as well as a marked inhibition of Fyn-dependent activation of reporter gene expression (16). Furthermore, Fyn protein levels were elevated in cells derived from $Cbl^{-/-}$ mice (16). Several SFKs, including Fyn, Src, Lck and Lyn, have been demonstrated to physically associate with Cbl, and Cbl is an excellent *in vitro* and *in vivo* substrate for these kinases (44-47). In contrast to Syk/ZAP-70, which interact with Cbl exclusively via its TKB domain, and RTKs, which require a Cbl TKB-mediated interaction for negative regulation, SFK regulation by Cbl is more complex. Previous studies have demonstrated that Cbl-SFK association involves binding between the SFK SH3 domain and the proline-rich sequences in the C-terminal half of Cbl (19). Furthermore, phosphorylated Cbl can also interact with the SH2 domain of SFKs (46), and an uncharacterized motif in Fyn can mediate its interaction with the TKB domain of Cbl (16). Consistent with these multiple modes of physical association, a TKB domain mutant of Cbl was fully capable of negatively regulating the levels and activity of Fyn; abrogation of Fyn SH3 binding to Cbl's proline-rich region, in addition to a Cbl TKB mutation, was required to block Cbl's effect on Fyn (16). Given these complexities of Cbl-SFK association, and the fact that two of these interactions involve the C-

terminal region of Cbl that is dispensable for EGFR and Syk/ZAP-70 regulation, it is essential to determine if Cbl-mediated negative regulation of SFKs indeed involves its core activity as a ubiquitin ligase.

Several recent studies have shown that certain SFKs are targets of ubiquitination (48-51). Blk, a B-cell specific SFK, was shown to interact with the ubiquitin ligase E6AP and undergo E6AP-dependent ubiquitination and degradation (48). Similarly, oncogenic v-Src and the wild-type c-Src expressed in CSK-deficient fibroblasts were shown to be ubiquitinated, and treatment with proteasome inhibitors led to increased protein levels (49,50). While the role of the Cbl protein family in the above situations has not been investigated, these findings are consistent with a hypothesis that Cbl regulates SFKs via ubiquitination, and that Cbl-mediated ubiquitination is a physiological mechanism to regulate the levels of activated SFKs.

Here, we have addressed this hypothesis through analyses of $Cbl^{+/+}$ and $Cbl^{-/-}$ cell lines, Chinese Hamster Ovary (CHO) cells with a temperature-sensitive defect in ubiquitin activating enzyme (E1), 293T cells co-expressing Cbl and its ubiquitination-deficient mutants, and *in vitro* ubiquitination assays. We provide direct evidence that Cbl negatively regulates the SFK Fyn by targeting it for ubiquitination, and that ubiquitination is a critical mechanism to regulate Fyn protein levels and activity. Given the conservation of structure between various SFK, and Cbl's ability to interact with multiple SFKs, we propose that Cbl-dependent ubiquitination provides a general mechanism to negatively regulate activated SFKs.

## EXPERIMENTAL PROCEDURES

*Cells* — 293T human embryonic epithelial kidney cells and primary embryo fibroblasts (PEFs) from wildtype ($Cbl^{+/+}$) and Cbl knockout ($Cbl^{-/-}$) mice were maintained as previously described (16). The CHO cell line CHO-TS20 (52), harboring a temperature-sensitive ubiquitin activating enzyme (E1), was kindly provided by Dr. G. J. Strous (University Medical Center Utrecht, Netherlands), and was maintained at 30°C in Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum (Hyclone), 20 mM HEPES pH 7.35, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies).

*Antibodies* —The following antibodies were used: monoclonal antibody (mAb) 12CA5 (anti-influenza hemagglutinin [HA] epitope tag; IgG2b) (53); mAb anti-ubiquitin (IgG1, MMS-258R) from Covance; rabbit polyclonal antibody (pAb) anti-p44/42 MAP kinase (9102) from New England BioLabs; mAb anti-EGFR (IgG2a, sc-120), pAb anti-Fyn (sc-16) and pAb anti-Cbl (sc-170) from Santa Cruz Biotechnology Inc.

*Expression plasmids*—The pSRαNeo-CD8-ζ chimera, Cbl and Fyn expression constructs in the pAlterMAX plasmid backbone (Promega) and GFP-Cbl expression constructs in the pCDNA3 vector backbone (Invitrogen) have been previously described (16,30,36,38). The EcoRI and SalI fragments of the respective pAlterMAX-Cbl constructs were cloned into pGEX4T-2-GST-Cbl-N (54) digested with EcoRI and XhoI to generate GST-Cbl constructs. The plasmid encoding HA-ubiquitin (55) was kindly provided by Dr. D. Bohmann (EMBO, Heidelberg, Germany).

*Transient Expression*— 293T cells were transfected as previously described using the calcium phosphate method (56). Cell lysates were prepared 48 hr post-transfection with lysis buffer (50 mM Tris pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 0.1% SDS and 0.05% DOC), supplemented with 1 mM phenylmethylsulfonyl, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 1 μg/ml each of leupeptin, pepstatin, antipain, and chymostatin.

TS20 cells were transfected for 6 hr using the Lipofectamine™ reagent in OPTI-MEM medium (Life Technologies), according to the manufacturer's protocol. The cells were cultured at 30°C for 56 hr, then either maintained at 30°C (permissive temperature for E1 function) or shifted to 42°C (non-permissive temperature). Cell monolayers were washed with cold phosphate-buffered saline and lysed on ice in the lysis buffer described above.

*Generation of Fyn-overexpressing PEFs*—PEFs overexpressing Fyn were established by retrovirus-mediated transfection of $Cbl^{+/+}$ and $Cbl^{-/-}$ PEFs. The MSCVpac retroviral vector (57) was kindly provided by Dr. R. G. Hawley (University of Toronto, Toronto, Canada) and the packaging plasmids pHIT60 and pMD.G were kindly provided by Dr. A. Y. Tsygankov (Temple University, Philadelphia, USA). MSCVpac-Fyn-T retroviral construct was generated by subcloning murine Fyn-T cDNA fragment from pAlterMAX-Fyn into EcoRI digested MSCVpac. Retroviral supernatants were produced as described and used to infect target cells (58). Bulk transfectant lines were selected in 5 ì g/ml puromycin (Sigma), and over-expression of Fyn was confirmed by anti-Fyn immunoblotting (Fig. 1A).

*Immunoprecipitation, gel electrophoresis and immunoblotting* — Immunoprecipitation was performed as described (54). The immunoprecipitated proteins and total cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidine difluoride (PVDF) membranes (NEN/DuPont), and immunoblotted with the indicated antibodies as described (32). Blots were visualized using the enhanced chemiluminescence detection system (NEN/DuPont). Blots were stripped and reprobed as described (37). Photographs were generated by direct scanning of films using a Hewlett Packard ScanJet 4c™ scanner.

*Pulse-Chase Analysis of Fyn Protein Turnover*—Fyn-overexpressing $Cbl^{+/+}$ and $Cbl^{-/-}$ PEFs were grown in 150-mm tissue culture dishes to about 70% confluence, rinsed with methionine-free DMEM (Life Technologies) and methionine-starved for 1 hr at 37°C in methionine-free DMEM supplemented with 2% dialyzed FBS (Life Technologies). Cells were then labeled for 1 hr at 37°C by supplementing the medium with 300 µCi/ml EXPRE$^{35}$S$^{35}$S labeling mix (NEN/DuPont). Cells were washed in DMEM, cultured in chase medium (DMEM supplemented with10% FCS and 3 mg/ml L-methionine) for the indicated times, and lysed in the lysis buffer described above. Anti-Fyn immunoprecipitations were performed on 1 mg protein aliquots of the cleared lysates. Bound proteins were resolved by SDS-PAGE, and gels were incubated in Entensify (NEN/DuPont) to enhance the radioactive signal. Radiolabeled proteins were detected by autoradiography of dried gels using BIOMAX-MR film (Eastman Kodak Co.). The signals were quantified by densitometric analysis of bands using ScionImage software (version beta 3b).

*Luciferase Assay*—293T cells were transfected by the calcium phosphate method with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Fyn constructs,

as previously described (16). At 48 hr post-transfection, cells were lysed with Cell Culture Lysis Reagent (Promega) and lysate protein concentrations were determined using the Bradford assay. Luciferase activity was determined on equal protein aliquots using a Monolight 3010C luminometer (Analytical Bioluminescence Laboratory Inc.) and Luciferin Reagent (Promega).

*In vitro Ubiquitination Assay*—GST-fusion proteins were affinity-purified on glutathione-sepharose beads from lysates of BL21(DE3)pLysE bacteria, as previously described (59). GST-fusion proteins were eluted using reduced gluthathione and dialyzed for 2 hr against 50 mM Tris/150 mM NaCl. GST-fusion proteins were further assessed for intactness and accuracy of quantification by SDS-PAGE followed by Coomassie staining. *In vitro* ubiquitination assays were perfomed on protein-A-sepharose bound Fyn or EGFR proteins obtained by immunoprecpitation from lysates of either transiently transfected 293T cells (Fyn) or A431 cells (EGFR) that were stimulated for 10 min with EGF (Sigma). The beads with bound Fyn or EGFR, were resuspended in ubiquitination reaction buffer (40 mM Tris pH 7.5, 5 mM $MgCl_2$, 2 mM DTT) (34) containing 10 μg of the indicated GST-Cbl fusion protein, 0.1 μg biotin-ubiquitin (Affiniti Research), and 5 μl crude rabbit reticulocyte lysate (Promega). The reactions were carried out for 1 hr at 30°C. The beads were then washed 6 times in lysis buffer, and bound Fyn or EGFR proteins were resolved by SDS-PAGE gel and transferred to PVDF membrane. Ubiquitin signal was detected by blotting with streptavidin-HRP (Zymed).

**RESULTS**

*Severely reduced ubiquitination of Fyn in PEFs derived from Cbl-deficient mice*—We have previously demonstrated that Cbl targets the Fyn protein for degradation in a 293T cell overexpression system (16). Consistent with a role for endogenous Cbl as a regulator of Fyn, we observed increased steady-state levels of Fyn and accumulation of phosphorylated Fyn in PEFs derived from $Cbl^{-/-}$ as compared to $Cbl^{+/+}$ mice. The latter system provided an opportunity to directly assess if endogenous Cbl controls Fyn ubiquitination. Given the relatively low levels of Fyn in PEFs and known difficulties in detecting endogenous ubiquitinated proteins (60), we used retroviral infection to establish a matched pair of $Cbl^{+/+}$ and $Cbl^{-/-}$ PEF lines expressing approximately ten-fold higher levels of Fyn compared to parental PEFs (Fig. 1A).

To establish if the presence or absence of Cbl in PEFs controls the stability of Fyn protein, as predicted by analyses in the 293T cell system, we performed a metabolic pulse-chase analysis of Fyn in transfected PEFs. Cells were pulse-labeled with $^{35}$S-methionine for 1 hr, followed by a chase in unlabelled methionine-supplemented media, and cell lysates were prepared at the indicated time points. Anti-Fyn immunoprecipitates of these lysates were resolved by SDS-PAGE and radiolabeled Fyn protein was visualized by autoradiography (Fig. 1B, *top panel*). Comparable $^{35}$S-Fyn signals were observed at time zero (no chase) in both cell lines (Fig. 1B, *top panel*, compare *lane 1* with *lane 5*). Whereas the radiolabeled Fyn signal in $Cbl^{+/+}$ PEFs showed a substantial time-dependent reduction of nearly 80% over the chase period, with a half-life of about 3 hr, Fyn protein in $Cbl^{-/-}$ cells was substantially more stable with only a small decrease in signal during the chase period (Fig. 1B and C). Immunoblotting of whole cell lysates with an anti-MAP kinase-specific antibody (Fig. 1B, *bottom panel*) revealed equal signals, confirming that differences in radiolabeled Fyn signals did not reflect a universal stabilization of proteins in $Cbl^{-/-}$ PEFs. These results established that endogenous Cbl controls the stability of the Fyn protein, and provided crucial reagents to directly assess if Cbl regulates the ubiquitination of Fyn.

To test the role of endogenous Cbl in Fyn ubiquitination, Fyn transfected $Cbl^{+/+}$ and $Cbl^{-/-}$ PEFs were incubated for 5 hr with (+) or without (-) the proteasome inhibitor MG132. Lysates of these cells were subjected to anti-Fyn immunoprecipitations followed by immunoblotting with an anti-ubiquitin antibody. A low but detectable ubiquitin signal, which appeared as a smear of slower migrating species similar to ubiquitinated species of other SFKs

(50,51), was observed in Fyn immunoprecipitates derived from lysates of $Cbl^{+/+}$ PEFs incubated in the absence of MG132; moreover, this signal was dramatically increased upon MG132 treatment of the cells (Fig. 1D, *top panel*, compare *lanes 1* with *lane 2*). In contrast, the ubiquitin signal was essentially undetectable in Fyn immunoprecipitates of lysates derived from $Cbl^{-/-}$ PEFs, and a very low ubiquitin signal was observed even after treatment with MG132 (Fig. 1D, *top panel*, compare *lane 2* with *lane 4*). Anti-Fyn immunoblotting of the same membrane showed that MG132 treatment of $Cbl^{+/+}$ but not $Cbl^{-/-}$ PEFs led to an increase in Fyn protein level (Fig. 1D, *bottom panel*). These findings directly demonstrate that Fyn protein undergoes ubiquitination and that the extent of Fyn ubiquitination is controlled by the level of endogenous Cbl protein.

*Intact cellular ubiquitination machinery is essential for Cbl-mediated Fyn degradation*— Stabilization of Fyn protein in $Cbl^{-/-}$ cells, together with accumulation of ubiquitinated Fyn in MG132-treated $Cbl^{+/+}$ cells, strongly suggested that Cbl-induced ubiquitination serves as a signal for proteasome-mediated degradation. To directly assess whether ubiquitination is required for Cbl-induced degradation of Fyn, we carried out further analyses in a CHO cell line (CHO-TS20) with a temperature-sensitive defect in the ubiquitination machinery. A thermolabile ubiquitin activating enzyme (E1) in these cells, which is fully active at 30°C but nonfunctional at 42°C (52), provided a convenient genetic system to manipulate the ubiquitination of Fyn using a temperature shift.

CHO-TS20 cells were transiently transfected with Fyn together with GFP-Cbl or a GFP control and maintained at the permissive temperature (30°C). The cells were then either maintained at the permissive temperature or shifted to the non-permissive temperature (42°C) for the indicated time points prior to lysis. Cell lysates were subjected to anti-Fyn immunoprecipitation followed by anti-ubiquitin immunoblotting to assess the level of Fyn ubiquitination. Very little Fyn ubiquitination was observed in the absence of co-transfected Cbl (Fig. 2A, *lanes 1-5*). In contrast, co-expression of Cbl caused a marked increase in the levels of ubiquitinated Fyn (Fig. 2A, *lane 6*). When cells were shifted to the non-permissive temperature, Fyn ubiquitination decreased rapidly; very little ubiquitinated Fyn was detected 9 hr after the temperature shift (Fig. 2A, compare *lane 6* with *lanes 9-10*). These results clearly demonstrated

that the level of Cbl-induced ubiquitination of Fyn could be precisely regulated in CHO-TS20 cells upon temperature shift.

The cell lysates used above were directly immunoblotted with anti-GFP and anti-Fyn antibodies to assess the levels of transfected Cbl and Fyn proteins. As anticipated, cells co-transfected with GFP-Cbl and Fyn showed a marked reduction in Fyn protein levels when compared to cells cotransfected with GFP vector (Fig. 2B, *middle panel*, compare *lane 1* with *lane 6*; densitometric units of 1.0 versus 0.1). When transfected cells were maintained at the permissive temperature (30°C), no substantial changes in the steady-state levels of Fyn protein were observed. In contrast, when Fyn plus GFP-Cbl co-transfected cells were shifted to the non-permissive temperature (42°C), a marked time-dependent increase in Fyn protein levels was observed (Fig. 2B, *middle panel, lanes 6-10*; densitometric units of 0.35 and 0.96 at 3 hr and 9 hr at 42°C versus 0.11 and 0.10 at 30°C, respectively). Relatively little change in Fyn protein level was observed when GFP and Fyn co-transfected cells were shifted to the non-permissive temperature (Fig. 2B, *middle panel, lanes1-5*; densitometric units of 1.04 and 0.97 at 3 hr and 9 hr at 42°C versus 0.87 and 1.09 at 30°C, respectively). Anti-MAP kinase immunoblotting of cell lysates revealed that changes in Fyn levels were specific and not reflective of general protein stabilization at 42°C (Fig. 2B, *bottom panel*). These results establish that Cbl-mediated degradation of Fyn requires intact cellular ubiquitination machinery.

*Cbl-mediated ubiquitination and degradation of Fyn requires the intact Cbl RING finger domain*—The results presented above are consistent with the hypothesis that Cbl targets Fyn for ubiquitination via its RING finger-domain encoded ubiquitin ligase activity. To address this possibility directly, we assessed the ability of the wildtype Cbl protein to target Fyn for ubiquitination in comparison with Cbl mutants in which the function of the RING finger domain has been disrupted. To assess Cbl-dependent Fyn ubiquitination *in vivo*, 293T cells were co-transfected with Fyn together with GFP or GFP-Cbl, and a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitinated Fyn. 48 hr post-transfection, the cells were treated for 5 hr with the proteasome inhibitor MG132 (+) or DMSO control (-). Cell lysates were subjected to anti-Fyn immunoprecipitations and immunoblotted with anti-HA antibody to detect ubiquitinated Fyn (Fig. 3A).

Transfection of GFP-Cbl led to easily detectable ubiquitination of co-expressed EGFR (used as a positive control); as expected (34), this signal was markedly enhanced by MG132 treatment (Fig. 3A, *top panel*, compare *lane 9* with *lane 10*). Relatively little ubiquitin signal was observed on Fyn in the absence of co-transfected Cbl. In contrast, co-expression of GFP-Cbl led to easily detectable ubiquitination of Fyn, which was accompanied by an expected decrease in the level of Fyn protein (Fig. 3A, compare *lane 1* with *lane 3*). MG132 treatment of cells prior to lysis resulted in marked accumulation of ubiquitinated Fyn and an increase in the level of Fyn protein (Fig. 3A, compare *lane 3* with *lane 4*). Equivalent expression of GFP-tagged Cbl protein in the appropriate lysates was confirmed by anti-GFP immunoblotting of whole cell lysates (Fig. 3A, *bottom panel*).

Next, we used the 293T cell system to examine the role of the RING finger domain-encoded ubiquitin ligase activity in Cbl-mediated ubiquitination of Fyn. The Cbl mutant C3AHN contains four point mutations that are predicted to prevent coordination of both zinc atoms in the RING finger domain (38,61), whereas the naturally occurring 70Z/3 Cbl mutant, which is unable to induce Fyn degradation (16), has a deletion of the critical linker region that connects the RING finger with the TKB domain (42). In contrast to wildtype Cbl, both 70Z/3 and the Cbl RING finger domain mutant C3AHN were unable to mediate Fyn ubiquitination (Fig. 3B, *top panel*) despite their equivalent or higher expression levels compared to wildtype Cbl (Fig. 3B, *bottom panel*). These findings indicated that the RING finger domain, and hence the ubiquitin ligase activity of Cbl, is necessary for Cbl-dependent ubiquitination of Fyn *in vivo*.

*In vitro reconstitution of Cbl-dependent Fyn ubiquitination and requirement of the Cbl RING finger domain*—We next tested if Cbl itself functions as a ubiquitin ligase towards Fyn. For this purpose, we tested the ability of recombinant Cbl proteins to mediate the ubiquitination of Fyn *in vitro*. Immunopurified Fyn protein bound to protein A-sepharose beads was subjected to *in vitro* ubiquitination in a mixture containing purified GST-Cbl proteins and biotin-labeled ubiquitin, as described in the methods section. Following the ubiquitination reaction, the bead-bound Fyn was washed, resolved by SDS-PAGE, and ubiquitin signals were detected by blotting with streptavidin-HRP. The validity of this assay was established by demonstrating the expected (34) GST-Cbl-induced, ATP-dependent ubiquitination of the EGFR immunoprecipitated from A431 cells, and lack of such activity with GST-Cbl-G306E and GST-Cbl-C3AHN (data not

shown). When *in vitro* ubiquitination reactions were carried out in the absence of Cbl, no ubiquitination of Fyn was detected (Fig. 4, *top panel, lane 3*). However, inclusion of recombinant Cbl in the reaction led to a dramatically high level of ubiquitinated Fyn (Fig. 4, *top panel*, compare *lane 3* with *lane 4*). In contrast, the Cbl RING finger mutant C3AHN was unable to mediate the ubiquitination of Fyn. Reblotting of this membrane with an anti-Fyn antibody revealed that enhanced ubiquitination in the presence of wild-type Cbl was accompanied by a decrease in Fyn protein level; however, there was no reduction in Fyn levels when the Cbl-C3AHN mutant was used (Fig. 4, *bottom panel*). These *in vitro* ubiquitination results provide compelling evidence that Fyn is a direct target of the ubiquitin ligase activity of Cbl and that a functional Cbl RING finger domain is required for this activity.

*The RING finger domain plays an essential role in Cbl-mediated negative regulation of Fyn-dependent cellular activation*—In order to determine the effect of Cbl-dependent ubiquitination on Fyn-mediated cellular activation, we compared the effects of wildtype Cbl and its RING finger domain mutants on Fyn kinase-dependent transactivation of the serum response element (SRE), linked to a luciferase reporter (62). 293T cells were transfected with the SRE-luciferase reporter plasmid and either Fyn alone or Fyn in combination with wildtype Cbl or its RING finger domain mutants. Each transfection was performed in replicates of five, and the luciferase activity was determined 48 hr post-transfection. As expected (16), the expression of Fyn protein led to a modest increase in SRE-luciferase activity compared to mock-transfected cells (Fig. 5A), and this increase was suppressed upon co-expression of wild-type Cbl. In contrast, co-expression of the Cbl RING finger mutant C3AHN as well as the 70Z/3 mutant (16) resulted in a marked enhancement of Fyn-dependent SRE-luciferase reporter activity (Fig. 5A). Expression of Cbl proteins without Fyn had no effect on the SRE luciferase activity. Analysis of cell lysates demonstrated the expected effects of Cbl proteins on Fyn protein levels and confirmed the equivalent expression of various Cbl constructs (Fig. 5B). Overall, these data demonstrate that the RING finger domain, which is required for Fyn ubiquitination and degradation, is also critical for functional negative regulation of Fyn by Cbl.

## DISCUSSION

The recently identified function of Cbl as a ubiquitin ligase (28,34), our earlier results that Cbl functions as a negative regulator of SFKs (16) and the emerging evidence that SFKs can be ubiquitinated (48-51), led us to hypothesize that Cbl ubiquitin ligase activity provides a physiological mechanism to control the levels of activated SFKs. Here we provide several lines of evidence in support of this hypothesis.

Analyses of multiple cell types, including primary embryonic fibroblasts, CHO-TS20 cells and 293T human embryonic kidney cells demonstrated the Cbl-dependent ubiquitination of Fyn. Similar results were obtained in a Jurkat T cell line stably overexpressing Cbl (37), where an accumulation of ubiquitinated Fyn was observed upon MG132 treatment (NR and HB, *unpublished results*). Importantly, we demonstrated that lack of endogenous Cbl leads to a drastic deficiency in Fyn ubiquitination in $Cbl^{-/-}$ PEFs. Reduced Fyn ubiquitination in $Cbl^{-/-}$ cells was accompanied by a substantial increase in endogenous Fyn levels (16) and a marked increase in the half-life of Fyn protein, indicating that Cbl-dependent ubiquitination was a critical determinant of Fyn turnover. It is notable that there are two other mammalian Cbl family members (18). Whether the lack of Fyn ubiquitination in $Cbl^{-/-}$ PEFs reflects a lack of expression of other Cbl family members or a lesser role for these proteins in Fyn ubiquitination will require further investigation.

A complimentary line of evidence for a critical role of Cbl-dependent ubiquitination in regulating Fyn protein levels was provided by analyses of CHO-TS20 cells, which express a thermolabile ubiquitin activating (E1) enzyme. This genetic approach provided further evidence that ubiquitin machinery is essential for Cbl to induce the degradation of Fyn. The results obtained in PEFs and CHO-TS20 cells clearly implicate the ubiquitin ligase activity of Cbl in negative regulation of Fyn. *In vivo* analysis in 293T cells, using Cbl RING finger domain mutants, established that this indeed was the case. Further analyses using *in vitro* ubiquitination assays demonstrated that Fyn was indeed a direct substrate for the ubiquitin ligase activity of recombinant Cbl. Taken together, our results establish Cbl-dependent ubiquitination as an important mechanism of negative regulation for Fyn, a prototype SFK. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among members of the SFK family, we propose that Cbl-dependent ubiquitination provides a general mechanism to negatively regulate activated SFKs.

It is likely that Cbl-mediated degradation functions in concert with other mechanisms for deactivation of SFKs, such as the return of activated SFKs to their repressed state through CSK-mediated phosphorylation of the C-terminal tyrosine and potential chaperone-mediated folding into a closed, inactive conformation. The ability of Cbl to target SFKs for ubiquitination and degradation also provides a likely explanation for why Cbl, unlike other SH3 domain ligands, does not activate SFKs. In contrast, SH3 domain ligands such as SIN (in the case of Src and Fyn) and the HIV Nef protein (in the case of Hck) enhanced the kinase activity of SFKs, with HIV Nef actually promoting oncogenic transformation by wild-type Hck (8,9,62). This proposal is supported by the ability of ubiquitin ligase-deficient Cbl mutants, such as 70Z/3 and C3AHN, to activate rather than downregulate SFK activity.

The proposed role of Cbl-mediated ubiquitination in SFK regulation is consistent with recent findings that other SFKs such as v-Src, c-Src, Lyn and Blk undergo ubiquitination (48-51). In the case of v-Src and c-Src, the ubiquitin ligase responsible for their ubiquitination has not been identified. As both v-Src and c-Src are known to interact with Cbl, it is likely that their ubiquitination may involve Cbl, although further analyses will be required to establish if this indeed is the case. Interestingly, ubiquitination of Blk was revealed through yeast-two hybrid studies that identified Blk as an interaction partner for the HECT domain-containing ubiquitin ligase E6AP. E6AP has been previously implicated in ubiquitin-dependent degradation of the nuclear tumor suppressor protein p53 by the human papilloma-virus oncoprotein E6 (63). Whether E6AP is a physiological ubiquitin ligase for Blk or other SFKs, and whether Cbl and E6AP might work in concert are obvious questions that will require further examination.

A number of observations suggest that Cbl-dependent ubiquitination and degradation primarily targets the activated pool of SFKs. The primary modes of physiological association between SFKs and Cbl involve the SH3 and SH2 domains of SFKs (16). These domains are intra-molecularly sequestered in repressed SFKs, and are predicted to be available for inter-molecular interactions only after activation. The additional interaction between Cbl and Fyn, mediated via Cbl's TKB domain, is also likely to involve an activation-dependent autophosphorylation site on Fyn and not the negative regulatory C-terminal phosphotyrosine, as Fyn Y528F mutant is highly susceptible to Cbl mediated degradation (16). Consistent with our proposal, the overall level of the autophosphorylated Fyn is markedly increased in $Cbl^{-/-}$ PEFs and T cell lines when compared to their $Cbl^{+/+}$ counterparts (16). In addition, we showed directly

that wild-type Cbl dramatically reduced whereas ubiquitination-defective Cbl mutants increased the Fyn-dependent activity of the SRE luciferase reporter, a known readout of the kinase activity of SFKs. Notably, co-expression of Cbl was also shown to reduce the Src-dependent induction of DNA synthesis in NIH 3T3 cells, and the inhibitory effect of Cbl was abrogated by deletion of the RING finger domain (17). Furthermore, ubiquitination of other SFKs such as Src and Blk, also correlated with their kinase activity (48-50). Previous findings have also established a correlation between increased kinase activity of SFKs and a reduction in their protein levels. For example, CSK-deficient cells have increased Src, Fyn and Lyn kinase activity but their protein levels are markedly reduced (12,13). It will be important to determine if reduction in SFK protein levels in these situations is Cbl-dependent.

While our results support a model that the major function of Cbl is to downregulate the level of activated SFKs by ubiquitin-mediated degradation, other studies have suggested that Cbl transduces signals downstream of SFKs. For example, several SFK-mediated cellular functions, such as integrin-induced macrophage spreading and bone resorption by osteoclasts, were severely reduced when cells were treated with Cbl antisense oligonucleotides (64,65). Furthermore, introduction of Cbl into v-abl transformed NIH 3T3 cells restored cell adhesion (58,66), and a truncated Cbl protein lacking the C-terminal region (Cbl 1-480), enhanced lamellipodia formation in transfected NIH 3T3 cells (67). It is therefore possible that Cbl can downregulate SFKs by targeting them for ubiquitination while simultaneously serving as an adapter for SH2 domain-containing proteins, thereby positively regulating signal transduction. In this regard, it is notable that the C-terminal phosphorylation sites of Cbl interact with the p85 subunit of PI3 kinase, the Rac/Rho exchange factor Vav and Crk adapter proteins (18), all of which are known to be involved in cytoskeletal remodeling, cell spreading and cell migration.

In conclusion, our results demonstrate that Cbl functions as a key regulator of the SFK Fyn by enhancing its ubiquitination and subsequent degradation via the proteasome. The negative regulatory role of Cbl is dependent on intact ubiquitin machinery as well as its RING finger domain, which is responsible for recruiting the ubiquitin machinery. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among various SFKs, we propose that Cbl-dependent ubiquitination provides a general mechanism to negatively regulate activated SFKs.

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**FOOTNOTES**

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Abbreviations used: CHO, Chinese hamster ovary; CSK, C-terminal Src kinase; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; GST, gluthathione S-transferase, HA, influenza hemagglutinin; HRP, horse radish peroxidase; IP, immunoprecipitate; mAb, monoclonal antibody; pAb, rabbit polyclonal antibody; PDGFR, platelet-derived growth factor; PEF, primary embryonic fibroblast; PTK, protein tyrosine kinase; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFK, Src-family kinase; SRE, serum response element; TKB, tyrosine kinase binding; WT, wild-type.

## FIGURE LEGENDS

FIG. 1. **Stabilization of Fyn protein and impaired Fyn ubiquitination in $Cbl^{-/-}$ primary embryonic fibroblasts.** *A,* Fyn protein levels in parental versus Fyn-transfected primary embryonic fibroblasts (PEFs). Equal amounts (50 μg) of protein lysates from the parental and Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ PEFs were resolved by SDS-PAGE and immunoblotted with anti-Fyn antibody. *B*, metabolic pulse-chase analysis of Fyn protein in Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ PEFs. $Cbl^{-/-}$ and $Cbl^{+/+}$ PEFs were methionine-starved for 1 hr and pulse-labeled with $^{35}S$-methionine for 1 hr, as described in materials and methods. The cells were washed and incubated in methionine-supplemented, unlabeled medium (chase) for the indicated times (hr, hours), and cell lysates were prepared. Anti-Fyn immunoprecipitates (IP) of cell lysates (1 mg) were resolved by SDS-PAGE, and labeled Fyn signals were detected by autoradiography (*top panel*). Equal amounts (50μg) of the same lysates were immunoblotted with anti-p42/44 MAPK antibody (*bottom panel*). *C*, the radioactive Fyn signals in *B* were quantified using densitometry, expressed as a percentage of the maximal signal intensity and plotted as a function of chase times. The line of best fit was calculated using the Prism program. *D*, impaired Fyn ubiquitination in $Cbl^{-/-}$ PEFs. Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ PEFs were incubated with 50 μM MG132 (+) or DMSO control (-) for 5 hr and then lysed. Anti-Fyn immunoprecipitates of 1 mg aliquots of lysate were immunoblotted with anti-ubiquitin antibody (*top panel*), and the membrane was stripped and reprobed with anti-Fyn antibody (*bottom panel*).

FIG. 2. **Cbl-mediated loss of Fyn protein requires intact cellular ubiquitination machinery.** *A*, impaired Fyn ubiquitination upon E1 inactivation in Cbl-transfected CHO-TS20 cells. CHO-TS20 cells were transfected with Fyn (0.2 ì g) expression plasmid together with 4 ì g of GFP or GFP-Cbl plasmids and incubated at 30°C for 56 hr. At this point (To), cells were either maintained at 30°C (30->30) or shifted to 42°C (30->42) for the indicated times. Anti-Fyn immunoprecipitates from aliquots of lysate protein (1 mg) were resolved by SDS-PAGE and immunoblotted with anti-ubiquitin antibody. *B*, stabilization of Fyn protein upon E1 inactivation in Cbl-transfected CHO-TS20 cells. Equal amounts (30 μg) of the same cell lysate used in *A* were resolved by SDS-PAGE and immunoblotted with anti-GFP antibody (*top panel*), anti-Fyn antibody (*middle panel*), and anti p42/44 MAPK antibody (*bottom panel*). The levels of Fyn

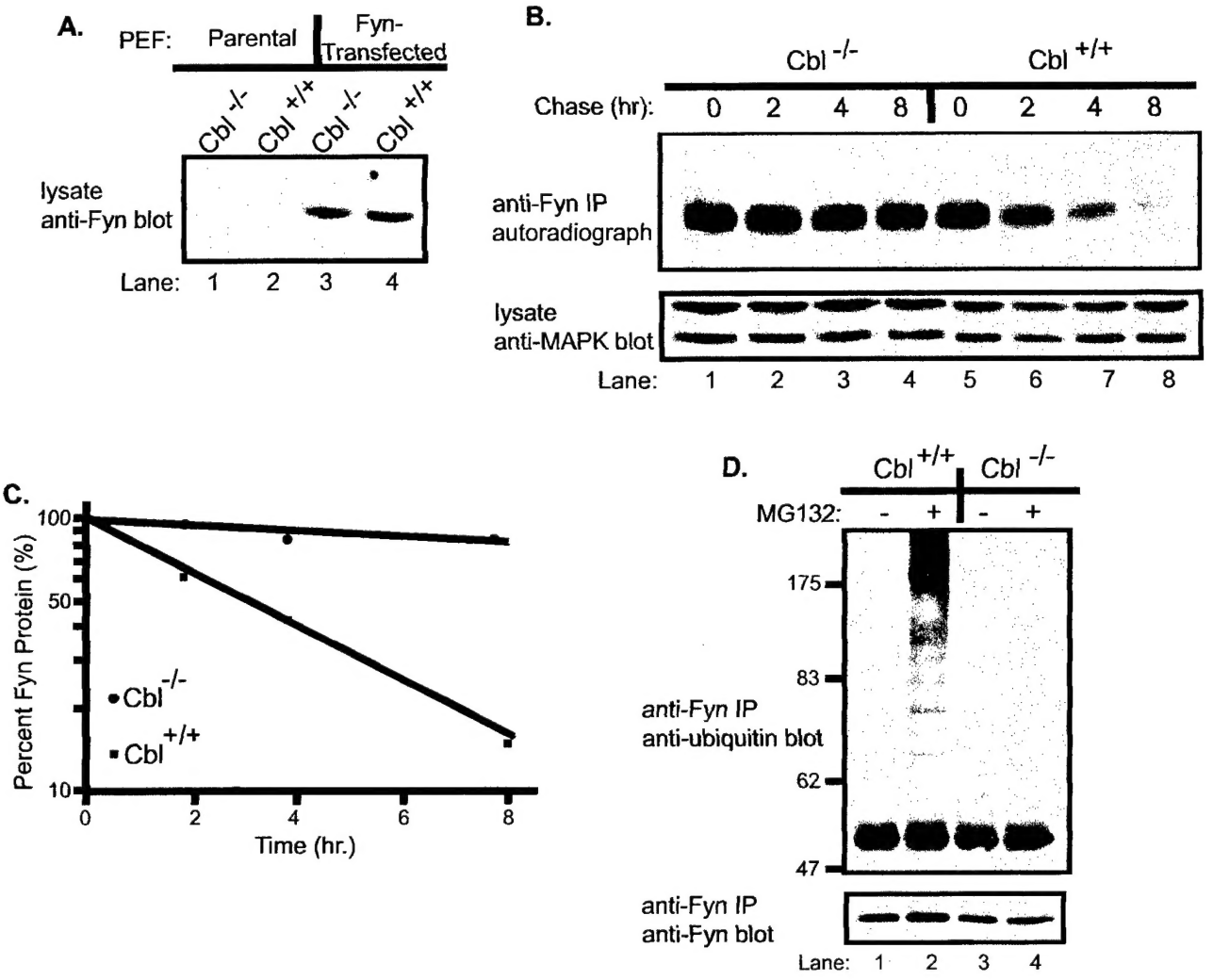
protein were quantified by densitometry, and the values at various times are expressed as a function of the initial Fyn protein level (*lane 1*) that was assigned a value of 1.0.

FIG. 3. **Cbl-dependent ubiquitination of Fyn in 293T cells and an essential role for the Cbl RING finger domain.** *A*, Cbl-dependent Fyn ubiquitination is enhanced by treatment with a proteasome inhibitor. 293T cells were transfected with plasmids encoding HA-ubiquitin (7 μg), Fyn (0.15 μg), EGFR (0.15 μg), GFP-Cbl (+) (3 μg) or a GFP (-) control (3 ì g). Five hr prior to cell lysate preparation, cells were treated with 50μM MG132 (+) or DMSO control (-). Anti-Fyn or anti-EGFR immunoprecipitates from aliquots of lysate protein (800 ì g) were resolved by SDS-PAGE and immunoblotted with anti-HA antibody (*top panel*). The blot was stripped and reprobed with anti-Fyn antibody (*middle panel*). Equal aliquots (30 μg) of the same cell lysates used above were resolved by SDS-PAGE and immunoblotted with anti-GFP antibody (*bottom panel*). *B*, an intact RING finger domain is required for Cbl-dependent Fyn ubiquitination. 293T cells were transfected with the indicated expression plasmids, lysed and anti-Fyn immunoprecipitations were carried out as in *A*. Immunoprecipitates and corresponding lysates (30 ì g) were resolved by SDS-PAGE. The immunoprecipitates were immunoblotted with anti-HA antibody (*top panel*) and with anti-Fyn antibody (*middle panel*). The lysate proteins were immunoblotted with anti-GFP antibody (*bottom panel*).

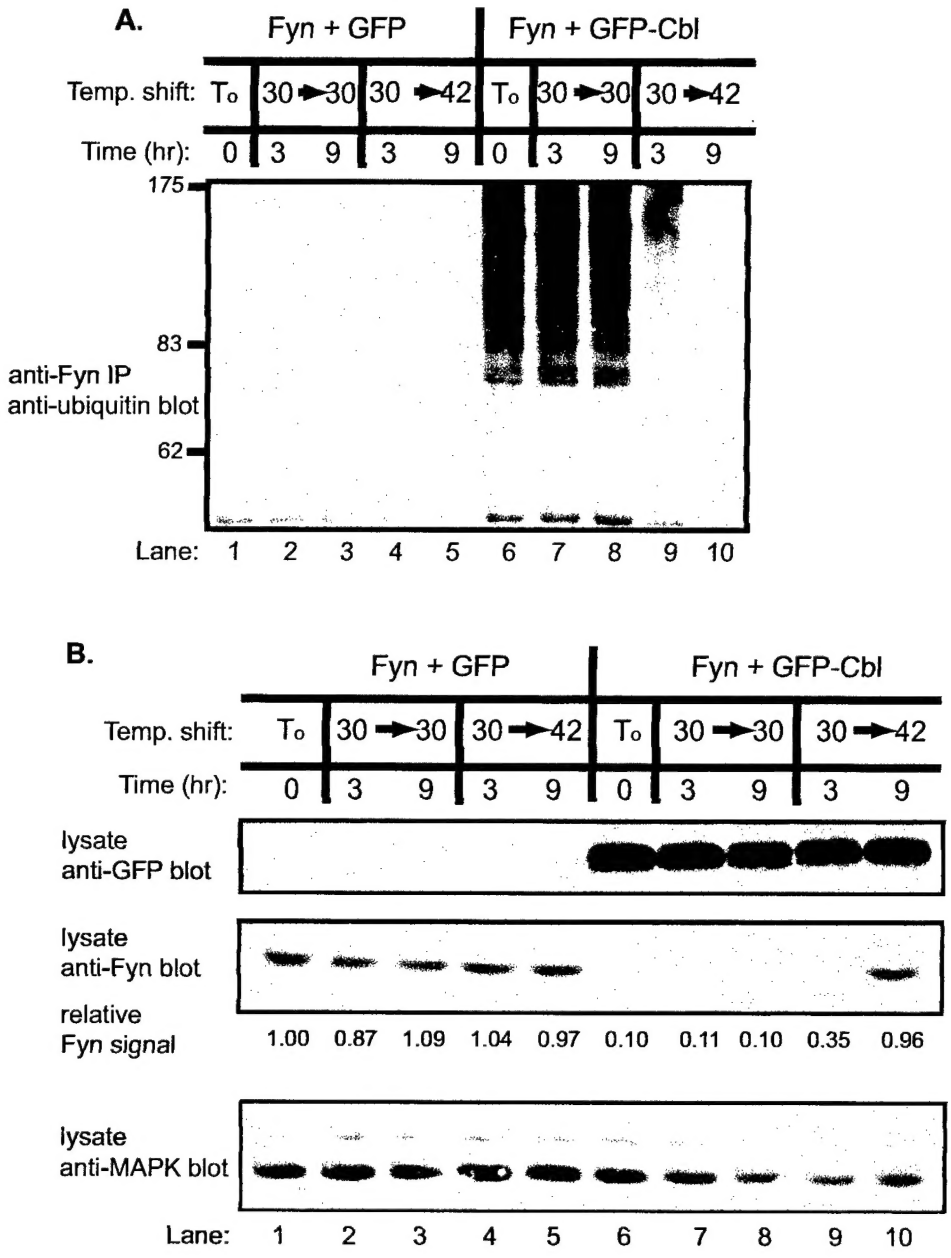
FIG. 4. **Direct *in vitro* ubiquitination of Fyn by recombinant Cbl requires an intact RING finger domain.** 293T cells were transiently transfected with a Fyn expression plasmid (+) or a vector control (-) as in Fig. 3, and anti-Fyn immunoprecipitates were carried out from aliquots of lysate protein (1 mg). After washing, the bead bound Fyn protein was incubated with the indicated GST-Cbl fusion proteins and biotin labeled ubiquitin in an *in vitro* ubiquitination reaction mixture (see Experimental Procedures). The bead-bound Fyn protein was washed, resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was immunoblotted with a streptavidin-HRP conjugate to visualize Fyn protein that had incorporated the biotin-labeled ubiquitin (*top panel*). The membrane was stripped and reprobed with an anti-Fyn antibody (*bottom panel*).

FIG. 5. **The RING finger domain is required for Cbl-dependent negative regulation of Fyn-induced transcriptional activation of a SRE-luciferase reporter.** *A*, Mutations in Cbl's RING finger domain blocks the negative regulation of SRE-luciferase activation. 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5 ì g), CD8-ζ (0.5 ì g), and the indicated combinations of Fyn (0.1 ì g), HA-Cbl, HA-70ZCbl and HA-C3AHNCbl (1 ì g) or pAlterMAX vector (-). Cells were lysed 48 hr after transfection and equal aliquots of lysate protein were used to assay luciferase activity. Luciferase activity was expressed relative to the luciferase activity of lysates transfected with the reporter in the absence of Fyn or Cbl. Results represent the mean +/- one standard deviation of five replicate transfections. *B*, analysis of Fyn protein levels in transfected cells used for SRE-luciferase assay. Aliquots of lysate protein (10 ì g) from two of the five replicate samples analyzed in *A* were resolved by SDS-PAGE and immunoblotted with anti-HA (top panel) and anti-Fyn (*bottom panel*) antibodies.

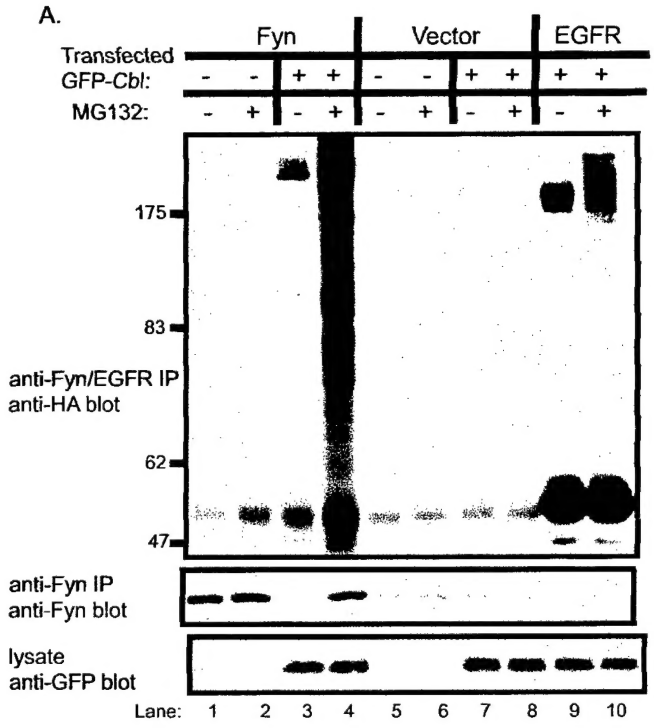
# Figure 1


A.
PEF: Parental
Fyn-Transfected
Cbl -/-
Cbl +/+
Cbl -/-
Cbl +/+
lysate
anti-Fyn blot
Lane: 1 2 3 4
B.
Cbl -/-
Cbl +/+
Chase (hr): 0 2 4 8 0 2 4 8
anti-Fyn IP
autoradiograph
lysate
anti-MAPK blot
Lane: 1 2 3 4 5 6 7 8
C.
Percent Fyn Protein (%)
100
50
10
•Cbl -/-
▪Cbl +/+
0 2 4 6 8
Time (hr.)
D.
Cbl +/+
Cbl -/-
MG132: - + - +
175
83
62
47
anti-Fyn IP
anti-ubiquitin blot
anti-Fyn IP
anti-Fyn blot
Lane: 1 2 3 4

# Figure 2


A.
Fyn + GFP
Fyn + GFP-Cbl
Temp. shift: To 30→30 30→42 To 30→30 30→42
Time (hr): 0 3 9 3 9 0 3 9 3 9
175
83
anti-Fyn IP
anti-ubiquitin blot
62
Lane: 1 2 3 4 5 6 7 8 9 10
B.
Fyn + GFP
Fyn + GFP-Cbl
Temp. shift: To 30→30 30→42 To 30→30 30→42
Time (hr): 0 3 9 3 9 0 3 9 3 9
lysate
anti-GFP blot
lysate
anti-Fyn blot
relative
Fyn signal
1.00 0.87 1.09 1.04 0.97 0.10 0.11 0.10 0.35 0.96
lysate
anti-MAPK blot
Lane: 1 2 3 4 5 6 7 8 9 10

# Figure 3


A.
Fyn
Vector
EGFR
Transfected
GFP-Cbl:
MG132:
175
83
anti-Fyn/EGFR IP
anti-HA blot
62
47
anti-Fyn IP
anti-Fyn blot
lysate
anti-GFP blot
Lane: 1 2 3 4 5 6 7 8 9 10

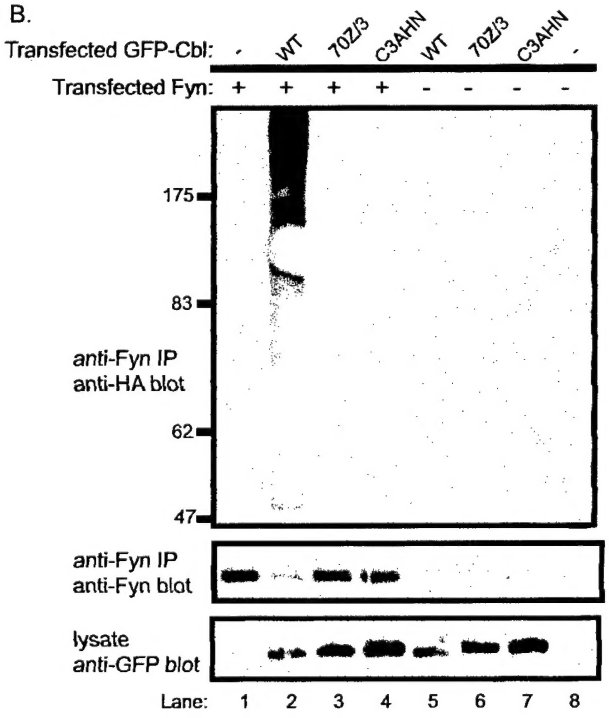

B.
Transfected GFP-Cbl:
WT
70Z/3
C3AHN
WT
70Z/3
C3AHN
Transfected Fyn:
175
83
anti-Fyn IP
anti-HA blot
62
47
anti-Fyn IP
anti-Fyn blot
lysate
anti-GFP blot
Lane: 1 2 3 4 5 6 7 8

Figure 4

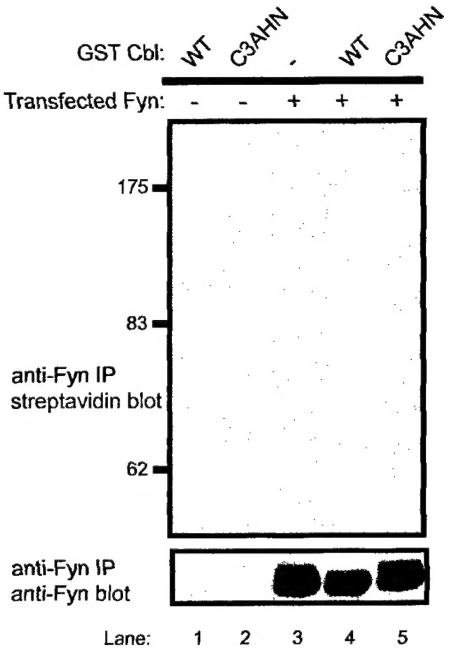



Figure 5